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Preliminary *in Vitro* Growth Cycle and Transmission Studies of HIV-1 in an Autologous Primary

W. P. TSAI,*‡,[2] S. R. CONLEY,† H. F. KUNG,* R. R. GARRITY,‡ and P. L. NARA‡

*Laboratory of Biochemical Physiology, Division of Basic Science and ‡Virus Biology Section, Directors Laboratory, Office of the Directors, Division of Basic Science, NCI-FCRDC, Frederick, Maryland 21702; and †Science Applications International, Frederick, Maryland 21702

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Recent interest focused on the dynamics of HIV-1 replication in primary monocytes/macrophages and T-lymphocytes of the immune system, as well as the standardization of virological and immunological *in vitro* assays with primary isolates, provided the impetus for these studies. These types of studies have never been performed as they would occur *in vivo,* i.e., where the envelope of the virus and cell membranes of the two cell types of the same host origin. Therefore, the biological and physicochemical properties of an uncloned, primary dual-tropic isolate HIV-1_{ADA} during the initial lag, log, and stationary phases of viral replication were studied in an autologous donor cell assay in peripheral blood mononuclear cells (PBMC) and blood monocyte-derived macrophages (MDM). Similar total numbers (10^9 virus particles/ml) were produced by both cell types during the stationary period. On a per cell per day basis, during peak stationary periods, 0.92×10^3 virions/day for MDMs and 5.31×10^3 virions/day for PBMCs were produced. Interestingly, virus replicating from MDMs during the log-growth phase demonstrated the greatest infectious fraction which was 3 logs greater than virus replicating in PBMCs. Despite constant virus particle production in MDMs, the infectious fraction was found to fall 3 to 4 logs over a 10-day period. Due to an infectious fraction less than 1 (0.053 infectious unit/cell/24 hr), virus spread in PBMCs during the rapid log phase could only have occurred by cell-to-cell contact, whereas in MDMs with an infectious fraction of about one infectious particle (~1/cell/24 hr), cell-free transmission could account for the observed results. Most of the MDMs (>90%) became productively infected, whereas only 5–10% of the total PBMCs were found replicating virus. The period of peak stationary virus production (i.e., stationary phase) was at minimum 4 to 5 times longer in MDMs than PBMCs. Whereas the majority of p24, RT, and gp120 found to be associated with MDM-derived virions, no increased dissociation of these components was observed in PBMC-derived virions. The virion-associated gp120 was 3 to 4 times more stable on both PBMC- and MDM-derived virus (>96 hr) and present at 10–25 times the concentration per virion than that observed for a T-cell-line-adapted laboratory strain of HIV-1 replicating in T-cell lines. These *in vitro* results suggest that important differences exist between MDMs and PBMCs with regard to the viral dynamics of infection and replication which should provide for a qualitative and quantitative basis to estimate virus replication on a per-cell basis for other known cellular targets of HIV-1. Studying the multiple biophysicochemical characteristics and viral replication dynamics as described herein provides an autologous *in vitro* model of additional quantifiable parameters for analysis and understanding of virus/host factor(s) and/or antivirals which influence them.

INTRODUCTION

Recent clinical virologic studies demonstrate that HIV-1 may prefer to replicate in various lineage-related macrophages at local portals of entry and/or in T-lymphocytes throughout the body early in infection and both of these cell types may constitute a major source and subsequent lymphoid reservoir of virus burden (for a review see Levy, 1993). CD4-bearing lymphocytes thus become infected during this initial lag-log phase of primary HIV-1 infection and are currently implicated in the maintenance of the plasma viral load and the subsequent high and rapid turnover of both CD4-positive cells. Virus replication dynamics and CD4 cell turnover have become the focus of ongoing rapid surrogate markers and efficacy determinants of antiretroviral therapy (Ho *et al.,* 1995; Wei *et al.,* 1995; Perelson *et al.,* 1996). Recent discussions have centered around further characterizing and defining the dynamics and turnover of virus replication, infected cells, and host controlling interaction(s) (Chun *et al.,* 1995; Ho *et al.,* 1995; Wei *et al.,* 1995; Levy *et al.,* 1996; Perelson *et al.,* 1996).

In these *in vivo* settings, both the viral envelope and the host cell membranes for the *de novo* infected cell types are from the same genetic origins. In addition, HIV-1 isolates appear to change following transmission and/or exhibit a spectrum of differential characteristics during disease progression in their biological and/or immunological properties such as replication capacity, cytopathic effect, ability to down-regulate CD4, tissue-specific host range/tropism, and susceptibility to sCD4 and

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[2] To whom correspondence and reprint requests should be addressed at Laboratory of Biochemical Physiology, NCI-FCRDC, P.O. Box B, Building 560, Room 12-15, Frederick, MD 21702-1201.

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neutralizing antibodies and/or cytotoxic T-cells as they are transmitted between macrophages, lymphocytes and/or other cells in the body (Nara *et al.,* 1992; Tsai *et al.,* 1992; Weiss, 1993; Levy, 1993 for reviews). These virological properties are greatly influenced in addition, by an array of intra- and extracellular biochemical and biophysical characteristics such as the differences in rates of replication versus spontaneous decay rate usually associated with the shedding of virus-associated gp120 and/or the loss of reverse transcriptase activity, the concentration/density of the viral envelope gp120 (Layne *et al.,* 1991, 1992), infectious to noninfectious ratios, defective interfering particles, and complexity of the viral quasi-species. These characteristics in turn may be influenced by the acquisition of various host/virus cell membranes which include a growing list (i.e., HLA-DR, DP, DQ, B2-microglobulin, ICAM-1, LFA-1, CD43, CD44, CD46, CD55, CD59, CD63, and the transferrin (CD71) (Arthur *et al.,* 1992; Cantin *et al.,* 1996; Montefiori *et al.,* 1994; Orentas and Hildreth, 1993; Saifuddin *et al.,* 1995) and plasma proteins (Nara *et al.,* 1991; Wu *et al.,* 1995).

Given the paucity of biochemical and biophysical data for primary isolates, and the need for more quantitative and qualitative *in vitro* data regarding the dynamics of virus replication, we have extended the previous studies (Layne *et al.,* 1991, 1992) to include a primary dual-tropic isolate. Furthermore, as HIV-1 has been found to incorporate various host cell proteins and replicates in an autologous cell setting *in vivo,* we designed an in vitro autologous cell assay in an attempt to better mimick those *in vivo* cellular conditions. Using growth cycle kinetic and wash-out protocols in MDM and PHA-stimulated PBMCs from the same normal donor (autologous), several properties of a primary isolate of HIV-1 such as replication rates, amounts of virus particles produced per cell, virion-associated gp120 density and spontaneous shedding rates, infectivity titrations, physical and biochemical particle enumeration, and infectious to noninfectious particle ratios were determined during the lag, log, and stationary growth phases. The results from these preliminary *in vitro* studies demonstrated that unique differences in the relationship between physicochemical and biological properties occurred during viral replication of a primary isolate of HIV-1 using an autologous primary cell system as described here. This report is the first to report such detailed *in vitro* viologic characterization utilizing an autologous cell assay system.

MATERIALS AND METHODS

Preparation of virus stocks

HIV-1 virus stocks were prepared using a uncloned, dual-tropic, primary cell passaged isolate, HIV-1_{ADA}, and a highly passaged uncloned CD4+ T-cell-tropic laboratory strain, HIV-1_{MN}. Co-cultured with rCSF-1-treated normal monocytes, HIV-1_{ADA} was originally isolated from PBMCs of an HIV-1 seropositive male homosexual with low CD4+ T cell counts (252 cells/mm^3) and Kaposi's sarcoma for 4 years from which a limited passaged isolate was obtained (Gendelman *et al.,* 1988). Titration of the working HIV-$1_{ADA/MDM}$ stock was carried out in rCSF-1-treated normal monocytes (cultured for 5 to 10 days before use) and the final $TCID_{50}$ was determined by end-point serial dilutions and measured by quantitative p24 ELISA at 2 weeks after infection. For this study HIV-$1_{ADA/MDM}$ was also passaged in PBMCs and the virus produced denoted as HIV-$1_{ADA/PBMC}$. The HIV-1_{MN} strain was originally isolated from PBMCs of a pediatric patient with AIDS-related complex by transmitting into JM T-cell line (Wong-Staal *et al.,* 1985) and subsequently maintained in the H9 transformed human T-cell line as HIV-$1_{MN/H9}$. The virus stock was prepared in H9 cells using an acute infection protocol as described previously (Layne *et al.,* 1991). For titration of HIV-$1_{MN/H9}$ virus stock, T-cell line CEM-SS was used as the indicator cells and the titers were expressed in syncytial-forming units (SFU), which were quantitated on Day 5 after infection (Nara *et al.,* 1987). The two virus stocks were then completely characterized biochemically and biophysically for total fractionated soluble and virion-associated p24 and total physical particles. To determine soluble and virion-associated p24, the virus stocks were ultracentrifuged and concentrations of p24 determined on both the pre- and postspin supernatants and viral pellets (summarized in Table 1). The virion concentrations as determined by electron microscopy (EM) and p24 for both stocks were within an order of magnitude with each other. These biochemical data served to characterize the input virus inocula and provided a basis from which to measure

TABLE 1

Characteristics of the HIV-1 Virus Stocks

	ADA[a]	MN[a]
Titer	10^7 $TCID_{50}$/ml	2×10^5 SFU/ml
p24 (ng/ml)		
Prespin	850.0	2400
Postspin	42.2	176
Pellet[b]	807.8	2224
Virion concentration in pellet (particles/ml) as determined by		
p24	16.2×10^9	44.5×10^9
EM	9.8×10^9	54.9×10^9

[a] HIV-1_{ADA} virus stock was made in rCSF-1 treated normal monocytes as described under Materials and Methods and then concentrated 100-fold by directly pelleting from culture medium. A different batch with 10-fold concentration prepared in similar fashion was used for the replication study. HIV-1_{MN} virus stock was made in H9. The stocks were provided by Dr. H. F. Gendelman.

[b] Calculated values: the difference between the prespin and postspin.

subsequent samples during the lag, log, and stationary phases in growth cycle presented later in the paper.

Isolation and culture of macrophages and PHA-activated PBMCs

The PHA-activated PBMCs and human monocytes/macrophages were both isolated from the same donor's pack (~275 ml) through leukapheresis and by a previously published methodology (Tsai *et al.,* 1991). The procedure consisted of the standard Ficoll–Hypaque gradient method followed by two sequential steps of adherence to plastics. The primary adherence was done with regular tissue culture flasks (polystyrene), followed by a hydrophobic plastic (polypropylene) adherence. Peripheral blood from healthy donors was first fractionated by the standard Ficoll–Hypaque gradient centrifugation procedures. The fractions of mononuclear cells (PBMC) were then pooled and washed, and erythrocytes were removed with a lysing buffer (ACK, Quality Biological, Inc., Gaithersburg, MD). PBMCs were then cultured in polystyrene plastic flasks (Costar) for 1 hr with RPMI medium plus 2% fetal bovine serum (FBS). The unattached cells were collected as the source of peripheral blood mononuclear cells (PBMC). The PBMCs were activated and cultured in RPMI 1640 with 10% FBS and phytohemagglutinin (PHA) (2 μg/ml) for 2 days, after which they were then changed to medium containing interleukin 2 (IL-2) (Hoffmann-La Roche Inc. provided through Biological Resources Branch, BRMP, DCT, NCI) at a concentration of 50 U/ml, and immediately infected.

The cells which adhered after the 1-hr incubation in 2% FBS described above were considered as crude preparations of monocytes/macrophages and were further cultured in RPMI with 5% FBS for additional 18–20 hr. The cells were then harvested, washed, and subjected to the second step of adherence by culturing in polypropylene in RPMI with 10% human AB type serum (Sigma) for 5 days. Unattached cells were removed and the adherent monolayer of cells were found to contain a highly pure population of differentiated macrophages as determined by electron microscopy, OKM1 staining, and nonspecific esterase activity (Sigma). The adherent macrophages could then be scraped off from the plastic and transferred to other tissue cultureware after incubation for 30 min in cold PBS with 1 m*M* EDTA. The cells were then used either for infection immediately or cryo-preserved as needed. A typical isolation from a pack of 275 ml of healthy human blood obtained by leukapheresis was found to contain approximately 6.4×10^9 total leukocytes with approximately 1×10^8 cells being MDMs. This method routinely yields roughly 1.5% of the total PBMCs obtained by this method (leukapheresis).

Biochemical characterization of HIV-1

Viral p24 antigen of the samples following treatment with Triton X-100 was determined by a commercial HIV-1_{IIIB} p24 ELISA kits (DuPont), previously quantitated and standardized by amino acid analysis using recombinant p24 (Layne *et al.,* 1992). Estimation of virion concentration from p24 concentrations was made based on the calculated and directly quantitated (direct particle counts and quantitative p24 ELISA) value of 5×10^{-8} ng of p24 per virion determined previously (Layne *et al.,* 1992).

The concentration of gp120 molecules per virion was initially determined using a modified gp120 ELISA, originally established by Moore and co-workers (Moore and Jarrett, 1988; Moore *et al.,* 1989). However, after finding that the human serum in the media for monocyte/macrophage cultures caused high background using this assay, a modified procedure was developed in which HIV-1 positive human IgG was directly conjugated with the enzyme. In brief, a gp120-specific carboxyl-terminal specific antibody coated on a microtiter plate was used to capture gp120. The captured antigens were then detected by a pool of directly conjugated HIV-1 positive human serum. Viral protein concentrations were determined against standard curves using recombinant HIV-1_{IIIB} gp120 derived from CHO cells (Celltech). Estimates of the total mass of virion-associated gp120 on a per virion basis were derived from dividing the pelleted virion gp120 concentration (g/ml) by the virion concentration (previously described above). The number of gp120 molecules per virion were then obtained by dividing total gp120 mass (g/virion) by 2×10^{-19} g/molecule, the mass of a gp120 molecule. The reverse transcriptase (RT) activity was measured by radioactive products produced by the reaction of the sample with tritiated TTP and oligo(dT) poly(A), as previously described (Layne *et al.,* 1992).

Quantitative electronmicroscopy

Direct particle counts of virus were carried out as previously described (Layne *et al.,* 1992). In brief, viral samples were mixed with polystyrene spheres which were identical in density/size to virus particles resulting in 1×10^9 spheres/ml. Multiple cross-sectional analysis of the viral/polystyrene bead pellet was done to assess a uniform distribution of beads and virus particles prior to counting. For each electron micrograph, total numbers of viral particles and polystyrene spheres were counted and four random electron-micrographs were used to determine the final virion concentration for a sample. Previous studies suggested the sensitivity of quantitative particle counts by EM require a minimum virus concentration of approximately 1.0×10^6 particles/ml. Reproducibility of this procedure has previously been determined (Layne *et al.,* 1992; Lin *et al.,* 1994) and repeated values fall within 0.25–0.50 logs at a 95% confidence interval.

Autologous primary MDM/PBMC assay

To better understand the *in vivo* relationship of viral replication, growth kinetics, infectivity, tropism, etc.,

where an autologous relationship exists between virus and host cells, we designed the primary autologous cell assay. Both 2-day, PHA-activated PBMCs and 5-day differentiated macrophages from the same donor were infected with HIV-1_{ADA} and HIV-1_{MN}. In both cases, a total of 18.6×10^6 cells were incubated with 1.1 ml of virus inoculum (obtained by mixing 1 volume of virus stock with 1.5 volume of medium) which resulted in a m.o.i. of 0.006 for the HIV-$1_{MN/H9}$ (as titered in CEM-SS cells) and 0.03 for the HIV-$1_{ADA/MDM}$ (titered in different donors macrophages). Following a 90-min incubation, the cells were washed three times and either resuspended in medium containing IL-2 for PBMCs or resuspended in RPMI1640 medium containing 10% human AB sera for MDMs. A total of 15×10^6 cells in 7.5 ml of medium (for both cell types) was then plated in T-25 flasks. Samples were then taken immediately (Day 0) for baseline determinations of p24, RT, and gp120 concentrations. On Day 2 following infection fresh media were added to both the PBMCs and MDM cultures to a final total volume of 11 ml.

To monitor kinetics of viral replication in both PBMCs and MDMs during the lag (early entry prior to detectable cell-free p24 detection), log-growth (exponential viral replication period), and stationary phases (peak sustained levels of viral replication), media were completely removed from the cultures and the cells extensively washed and replaced with 11 ml of fresh media containing 20–30% conditioned RPMI 1640. This wash-out procedure was repeated every 3 or 4 days throughout the lag, log, and stationary phases which lasted (depending on the primary cell type) over a 10-day to 5-week period. Samples for virus characterization were separated by ultracentrifugation into viral pellets and prespin and postspin supernates and assayed in all fractions for p24, gp120, RT activity, and infectivity titrations as well as particle counts by electron microscopy. The extensive characterization as described previously, and cost associated with all these determinations, prevented us from repeating the experiment with more than one HIV-1 isolate and one normal donor's cells. Repeat experiments of the viral replication kinetics studies measuring p24 were subsequently performed for HIV-$1_{ADA/MDM}$ on different donors' cells as part of other studies (Tsai, in preparation) and found to be similar to those observed in this study.

To further characterize the relationship of infectivity of HIV-1_{ADA} between MDMs and PBMC's of the same donor, with virus replicating in that donor's cells, infectivity titrations were done back on the same donor's MDMs and PBMCs (autologous cell assay). Serial kinetic samples taken from the HIV-1 infected MDMs and PBMCs above were titered back on the same donor's PBMCs and MDMs using 0.1 ml of the sample with a 10-fold dilution series in duplicate and placed into 96-well microtiter plates containing preplated uninfected primary cells and incubated for 1 hr.

In addition, the contribution of infectious virus within the intracellular MDM compartment was studied in a fashion similar to that for the replication rate study described above, and the experiment was carried out in 48-well-plates at 3×10^5 cells/well. The cells were scraped from the wells 14 days after the infection, washed extensively, placed in medium and disrupted by three cycles of freeze/thaw with an ethanol/dry ice bath. The resulting cell lysates were then titrated for the infectivity as described above.

RESULTS

Replication kinetics, biochemical characteristics, and infectivity of HIV-1_{ADA} in MDMs and PBMCs

The dual-tropic primary isolate HIV-1_{ADA} was passaged through both MDMs and PBMCs to characterize various virological, biochemical, and biophysical properties. These include replication kinetics, the effect of host cell passage, the quantitation of both soluble and virion-associated major structural proteins (p24, RT, and gp120), infectivity, virus particle quantitation as determined by both physical particle counts (EM) and the concentration of structural protein p24, and finally the infective/noninfective ratios. The replication kinetics of HIV-1_{ADA} on macrophages as measured by p24 antigen, RT activity, and gp120 antigen in the culture media are shown in Figs. 1A, 1B, and 1C, respectively. The lag phase of virus replication in MDMs, (as defined to include eclipse time and time for the virus to spread) was observed by the declining values of corresponding p24 (Fig. 1A) and virion concentrations (Table 2) by 7 days, after which, replication of the new virus was observed to occur rapidly between Days 10 and 14 in log fashion reaching peak stationary levels by Day 17 and continuing unabated to Day 29. Approximately 300 ng/ml of p24 (Fig. 1A) or 5.8×10^9 HIV-1 particles (Table 2) were made every 3–4 days during the log and stationary phase (Day 14 to Day 24). The majority of viral proteins such as p24 antigens (~99%), RT activity (100%), and gp120 antigens (96%) were associated with the virus pellets following ultracentrifugation of the clarified culture media (Figs. 1B and 1C). In individual samples taken from the log phase (Days 10–14 and 14–17), gp120 was associated with the virus pellet, whereas at Day 20 a small amount of both soluble p24 (~1%) and gp120 (4%) was detected in the supernatant (Figs. 1A and 1C and Table 2). The virus particle count calculated from p24 concentration above was further confirmed by quantitative EM. The cell-free HIV-1_{ADA} concentrations produced by MDMs ranged from 3.0×10^7 particles/ml (Day 10) to 6×10^9 particles/ml (Day 20). These values closely agreed (within twofold) with those determined on Days 20 and 24 by EM (Table 2). Similar kinetics of RT activity (Fig. 1B) or gp120 antigens (Fig. 1C) coincided with viral replication as measured by p24 antigen.

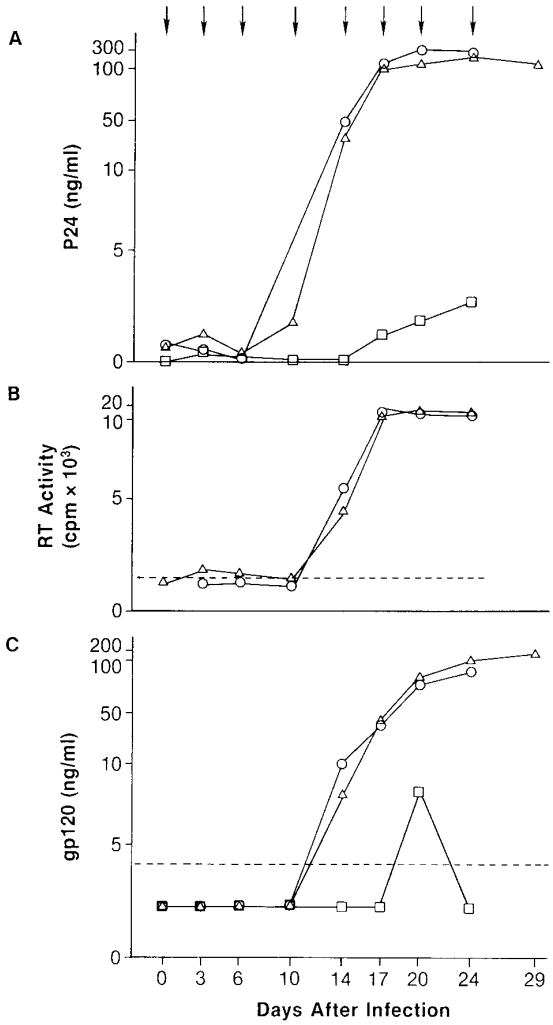

FIG. 1. Replication of HIV-1_{ADA} in human MDMs. The infection was carried out as described under Materials and Methods. Culture medium was completely collected every 3 or 4 days after infection and subsequently fractionated by ultracentrifugation. The viruses were monitored by determination of p24 antigens (A) reverse transcriptase (RT) activity (B) and gp120 antigens (C). The antigen/RT activity was determined in supernatant fluids before (-△-) and after (-□-) ultracentrifugation, and in the pellets (-○-). The dot line (---) indicates the low limits of the assays. The arrow (↓) indicates the time point when the culture medium was collected and cultures were washed three times immediately after.

Levels of RT activity and gp120 antigens in these cultures remained undetectable 5 days longer than p24 concentrations following infection due to assay sensitivities.

The cytopathic effects associated with active replication were mainly the formation of multinucleated giant cells. The MDM culture showed small syncytia (~5–10 nuclei per cell) within a week after infection, which further developed into giant syncytia (~50–100 nuclei per cell) after 2 to 3 weeks of infection and were found to be viable for the period of experimental observation (data not shown). EM and immunohistochemical staining revealed that extracellular virus in this culture system was produced from both single and multinucleated giant cells (data not shown). Also, no intracellular infectious virus could be demonstrated (data not shown).

For comparison, the uncloned HIV-$1_{ADA/MDM}$ was also similarly characterized in autologous PHA-stimulated PBMCs at the same m.o.i. of 0.03 (Figs. 2A, 2B, and 2C). Replicating virus, as measured by pelletable p24 antigens (Fig. 2A) appeared within 3 days after infection (log phase). Wash-out experiments done between Day 3 and Day 6 (arrows) demonstrated an exponential increase of both p24 and gp120 from 2 to 150 ng/ml and 1 to 20 ng/ml, respectively (Figs. 2A and 2C). Replication reached a peak level by Day 10 (~200 ng/ml p24), and declined over fivefold by Day 14. RT activity and gp120 antigen were undetectable until Day 6 (3 days later than p24), after which similar kinetics as described previously were observed (Figs. 2B and 2C). The p24 taken between Day 3 and Day 6 was mostly associated with the virus pellet (80%). Greater amounts of gp120 (~90%), however, were found in the pellet after ultracentrifugation. RT determined during the same time (testing both the total and the virus pellet) demonstrated that 50% of the activity was soluble. In the following washout interval (Days 6–10), however, approximately (within the error of the assay) 100% of the RT activity and 100% of the p24 and gp120 antigens were found associated with the PBMC virus pellet (Fig. 2B). Thus, no increased dissociation of p24, RT, and gp120 was observed with virions produced by either PBMCs or MDMs.

A shorter stationary phase of virus production than in MDMs was observed for PBMCs lasting only 4 days (Days 6 and 10, arrows). Approximately 200 ng/ml p24 (Fig. 2A) or 3.8×10^9 particles of HIV-1 were made within 4 days (Table 2). Quantitation of virion production by direct particle counts yielded similar values, 3.5×10^9 particles/ml, which were in close agreement with the p24 estimates (Table 2). The decline of virus production observed in these cultures after Day 10 was probably due in part to loss of cell viability (Fig. 2D).

To contrast the growth characteristics of cell line-adapted HIV-1 in primary cultures with HIV-1_{ADA} as shown above, HIV-$1_{MN/H9}$ was used to infect both PBMCs and MDMs derived from the same donor as for the HIV-1_{ADA} virus studies. The HIV-1_{MN} showed a similar short log period followed by virus replication in PBMCs, as measured by p24 antigens (Fig. 3A). However, unlike HIV-1_{ADA} in PBMCs, a sustained plateau phase of virus replication was observed to Day 14. Overall, lower virus replication (~8- to 10-fold lower) and peak levels (25 ng/ml) were observed through Day 14. The majority of p24 (~97%) was also associated with pellets (Fig. 3A) and only a small amount (~3%) was left in the supernatants after centrifugation of the culture medium. The concentra-

TABLE 2

Comparison of Biophysical, Biochemical, and Biological Properties of HIV-1_{ADA} and HIV-1_{MN} Viruses Replicated in MDMs and PBMCs

Virus	Property[a]	Days after infection 0	3	6	10	14	17	20	24	29
ADA made in MDM	Virion concentration ($\times 10^6$/ml)									
	p24	11.4	6.4	1.4	30[b]	970	3075	5870	5080	2950[b]
	EM	ND[c]	ND	ND	ND	ND	ND	2400	3900	ND
	gp120 (molecules/virion)	L[d]	L	L	ND	55	190	190	90	ND
	RT activity	L	L	L	L	72	73	36	41	ND
	End point titers determined in									
	MDM	<1	2	<1	4	>5	4	2	3	ND
	I/NI Ratio	NV[e]	10^{-5}	NV	10^{-3}	10^{-4}	10^{-5}	10^{-7}	10^{-6}	
	PBMC	<1	<1	<1	2	4	>5	3	1	ND
	I/NI ratio	NV	NV	NV	10^{-5}	10^{-5}	10^{-4}	10^{-6}	10^{-8}	
ADA made in PBMC	Virion concentration ($\times 10^6$/ml)									
	p24	6.3	32.0	2290	3830	731				
	EM	ND	ND	ND	3490	ND				
	gp120 (molecules/virion)	L	L	50	210	50				
	RT activity	L	L	28	23	11				
	End-point titers determined in									
	MDM	1	2	3	2	2				
	I/NI ratio	10^{-5}	10^{-5}	10^{-6}	10^{-7}	10^{-7}				
	PBMC	1	2	3	2	2				
	I/NI ratio	10^{-5}	10^{-5}	10^{-6}	10^{-7}	10^{-7}				
MN made in PBMC	Virion concentration									
	p24	5.9	79.0	22.0	351.0	507				
	EM	ND	ND	ND	ND	1950				
	gp120 (molecules/virion)	L	L	L	250	190				

[a] Virion concentration (10^6 virions/ml) in pellets determined by p24 and electromicroscopy (EM). gp120 concentration (gp120 molecules/virion) calculated as the virion concentration determined by p24 concentration. RT activity (counts per minute per 10^6 virions determined by p24 concentration). Titers determined by detection of p24 antigens: the prespin samples were diluted in 10-fold series and titrated separately in MDMs and PBMCs in microtiter plates, and the titers/ml are expressed as a logarithm of the highest dilutions that showed p24 positive. I/NI ratio, ratio of titers to virion concentrations determined by p24.

[b] In prespin culture medium.

[c] Not done.

[d] L, below the lower limit.

[e] NV, No values.

tion of virus particles as determined by both p24 and EM was 0.5×10^9 per milliliter and 2.0×10^9 per milliliter, respectively (Table 2). The RT activity and gp120 concentrations were below detectable limits up to Day 14 and Day 10, respectively (Figs. 3B and 3C). The HIV-$1_{MN/H9}$ strain did not productively replicate in MDMs (data not shown).

Estimation of virus production and rate on a per cell basis

Cell-free virus production during the stationary period was determined on a per cell basis for both PBMCs and MDMs from the relationship between the cell concentration per T25 flask (15×10^6 total macrophages in 11 ml of medium) and the amount of virus produced per unit time following a complete wash-out of cell-free virus. The average amount of the virus produced in MDMs infected with HIV-1_{ADA} between Day 20 and Day 24 was 9.2×10^9 virions/10^7 cells/day or 0.92×10^3 virions/cell/day, assuming that all MDMs are infected. Repeat assays demonstrated that greater than 90% of the MDM monolayer was infected as confirmed by p24 immunohistochemistry as early as 10 days (data not shown). Virion concentrations were derived from the average determinations measured by p24 and EM and found to be 11.7×10^2 and 6.6×10^2 particles per cell, respectively (Table 2).

The same values at the same growth periods were determined for HIV-1_{ADA} replicating in PBMCs between Day 6 and Day 10 (Figs. 2A and 2D). During the stationary phase, the total cell number of 2.20×10^6 viable PBMC

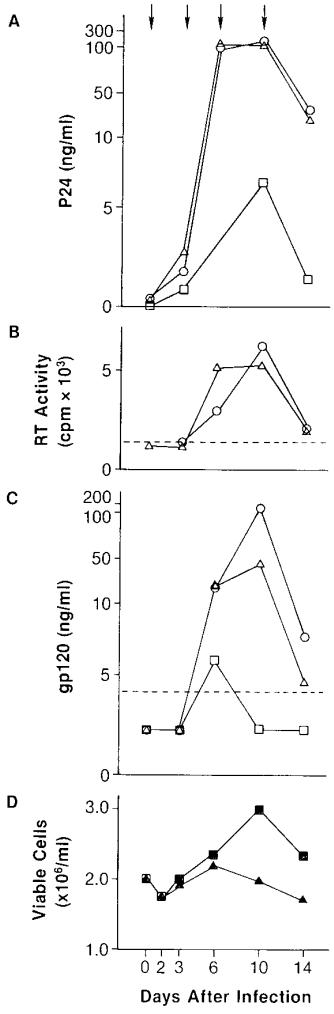

FIG. 2. Replication of HIV-1_{ADA} in human PBMCs and growth kinetics of PBMCs. The viruses were monitored by the assays as shown in Fig. 1 (A, B, and C). (D) Growth kinetics of infected PBMCs (-▲-) and uninfected controls (-■-).

cells/ml was reduced by 200,000 cells/ml to a nadir of2.00 $\times$ 10^6 viable cells/ml at Day 10. Direct sampling of cell counts and viability as well as independent HIV-1 specific immunohistochemical studies demonstrated that 5–10% of the cells were infected and lost during the short stationary phase of virus production (data not shown). The virion concentration of 3.66 $\times$ 10^9 particles/ml used in the calculation was the accumulated value collected over a 4-day interval following a complete washout of cell-free virus at Day 6. This value was determined from the average between both the pelleted p24 value (3.83 $\times$ 10^9/ml) and the EM count (3.49 $\times$ 10^9/ml) (Table 2). Therefore, the amount of virus produced on a per cell basis (assuming 8% of the PBMC infected) was approximately 5.31 $\times$ 10^3 virions/cell/day.

The replication of the laboratory strain HIV-$1_{MN/H9}$ in PBMCs produced two- to eightfold less total virions during the relatively noncytopathic peak stationary period of 14 days than HIV-$1_{ADA/MDM}$ in PBMCs (Table 2).

Estimates of virion-associated gp120, infectivity, and infective/noninfective particle ratios

In an effort to further characterize biochemical similarities or differences possibly affecting virion infectivity, tropism and neutralization resulting from the replication in primary cells, soluble and virion-associated gp120 concentrations, infectious titers and infective to noninfective particle ratios (I/NI) were also determined. Virion-associated gp120 concentrations from the same virus supernate/pellet samples described above (infecting MDMs) exhibited a log-linear increase in concentration from 10 ng/ml on Day 14 to 80 and 100 ng/ml at Days 20 and 24, respectively (Fig. 1C). Concentrations of virion-associated gp120 from HIV-1_{ADA} infecting PBMCs were measurable at Day 6 (~20 ng/ml) and peaked at Day 10

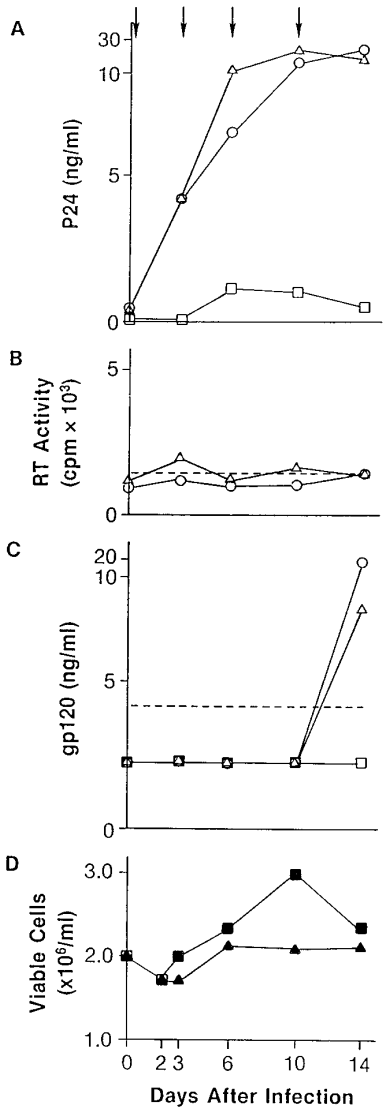

FIG. 3. Replication of HIV-1_{MN} in human PBMCs and growth kinetics of PBMCs. The viruses were monitored by the assays as shown in Fig. 1. (A, B and C). (D) Growth kinetics of infected PBMCs (-▲-), and uninfected controls (-■-).

(~100 ng/ml) (Fig. 2C). A large portion of the total gp120 (96–100%) for HIV-1_{ADA} measured from either MDMs or PBMCs appeared to be associated with the virus pellet (Figs. 1C and 2C). When calculated on a per virion basis, the gp120 concentrations ranged from 55 to 190 molecules per virion and from 50 to 210 molecules per virion from HIV-1_{ADA} in MDMs and PBMCs, respectively (Table 2). Similar concentrations of virion-associated gp120 were observed for HIV-$1_{MN/H9}$ replicating in PBMCs and ranged from a high of 250 molecules per virion on Day 10 to 190 molecules on Day 14 (Table 2). In contrast, the same HIV-$1_{MN/H9}$ virus in two separate stocks derived in H9 cells generated over a 2-year period demonstrated the typical lower virion-associated gp120 concentrations of 2.0 and 6.0 molecules per virion (data not shown).

Samples taken from HIV-1_{ADA} during the log growth period in MDMs were tested for resultant infectivity on both MDMs and PBMCs from the same donor. Initially, a 2-log titer on MDMs (Day 3) disappeared on Day 6 and reached peak levels (greater than 5 logs) by Day 14. Infectivity for MDMs declined to a level of 2 and 3 logs per milliliter by Day 20 to Day 24, respectively (Table 2). Infectivity of these same samples, when tested on the same donor's PBMCs, was negative at both Day 3 and Day 6, after which 2 logs of infectivity on Day 10 and greater than 5 logs of infectivity on Day 17 were observed (Table 2). These titers then declined from 3 logs on Day 20 to 1 log by Day 24 (Table 2). Serial samples from the same time course experiments with HIV-$1_{ADA/PBMC}$ demonstrated a more consistent pattern of infectivity (1 to 3 logs), which was similar on both MDMs and PBMCs (same donor). Both PBMCs and MDMs displayed the same infectious titers 10^2 (Day 3) which peaked to 10^3 (Day 6) and declined by 1 log by Day 10 (Table 2). In general, at least 3 logs of infectivity were present in all supernates tested before they became positive in the gp120 ELISA (4 ng/ml cutoff).

HIV-$1_{MN}/_{H9}$ replicating in PBMCs demonstrated lower and slower viral replication as evidenced in the delayed appearance of virion-associated gp120 (20 ng/ml on Day 14). No soluble gp120 was measured in the postspin supernate demonstrating the concentration was less than the detectable cutoff value of 4 ng/ml (Fig. 3C).

The infectious titers for HIV-$1_{ADA/MDM}$ and HIV-$1_{ADA/PBMC}$ were determined directly and reciprocally on the same donor MDMs and PBMCs as described above. The I/NI ratios during the growth cycle studies were then calculated by a division of the mean infectious titer with the virion concentration. The I/NI ratio of HIV-$1_{ADA/MDM}$ titered on MDMs exhibited a dynamic range from $1{:}10^3$ on Day 10 to a value of $1{:}10^7$ on Day 20, whereas, when titered on PBMCs, a rather constant I/NI ratio was observed ranging from $1{:}10^5$ on Day 10 to a value of $1{:}10^6$ on Day 20 (Table 2). The I/NI ratio for HIV-$1_{ADA/PBMC}$ titered back on PBMCs and MDMs demonstrated a range from $1{:}10^5$ on Day 3 to a value of $1{:}10^7$ on Day 10 (Table 2).

DISCUSSION

In this study a variety of biophysical, biochemical, and biological properties including replication kinetics were determined for a primary, dual-tropic isolate of HIV-1 infecting autologous primary cultures of human MDMs and PBMCs. Growth cycle studies performed as time course with the wash-out procedure permitted for the qualitative and quantitative characterization of a primary isolate of HIV-1 during the initial lag, log-growth and stationary phases of viral replication and extends our previous studies using a laboratory strain of HIV-1 (Layne *et al.,* 1991, 1992). To date, no such quantitative and qualitative data of this nature have been reported for a dual-tropic primary isolate in reciprocal and autologous primary cell cultures. By monitoring viral proteins p24, RT, and gp120 envelope antigens in combination with direct virus particle counts and virus infectivity, we determined the concentrations and ratios of I/NI particles in medium and rates of virion production on a per cell basis, as well as the density and relative stability of gp120 molecules on a virion.

In both PHA-activated PBMC and differentiated MDM cultures infected with the same m.o.i. of HIV-$1_{ADA/MDM}$ resulted in a significant level of newly synthesized extracellular p24 after a lag phase of 0 to 3 days and 6 to 10 days, respectively. Following a lag phase, virus replication increased rapidly by two to three orders of magnitude in both PBMCs and MDMs (log phase) by Day 6 and Day 17, respectively. MDMs as studied in this report demonstrated peak virus production during the stationary phase for the entire experimental period lasting a total of more than 2 weeks (data not shown). The peak stationary period of viral replication for PBMCs, on the other hand, began to decline after only 4 days, apparently due in part to loss of cell viability. During these growth cycle studies both primary cell types produced similar concentrations (~10^9 particles per milliliter) during their respective peak stationary periods. On a per cell basis, an average of 0.92×10^3 and 5.31×10^3 virions were produced daily (i.e., 24 hr), respectively, by an infected MDM and a PHA-activated lymphocyte in the culture flask. Interestingly, despite their smaller size, PBMCs were capable of producing 0.7 logs more virus (4400 more virions per cell per day) than MDMs. This may, in part, explain a more direct cytopathic effect for the virus on CD4 lymphocytes and the prolonged viability of HIV-1 infected MDMs, as the relative amounts of cell membrane involved in virus production would be significantly greater for a lymphocyte than a macrophage.

Previous estimates of rates and amounts of virus per cell have been done using laboratory strains in either PBMCs and/or T-cell lines and reported to be 10^2 to 10^4 physical particles (McDougal *et al.,* 1985; Volskey *et al.,* 1990; Dimitrov *et al.,* 1993; and Levy *et al.,* 1996). The greater values (one log) observed in this study than pre-

viously reported for HIV-1 in PBMCs (Levy *et al.,* 1996) may reflect improved *in vitro* differences in replication efficiencies inherent for a given primary isolate in primary cells and/or other variables (i.e., cell concentrations, cell culture conditions, as well as quantitative methodologies employed). Interestingly, whereas most of the MDMs and human T-cell lines became infected (>90%), only 5–10% of the PBMCs were found infected similar to a result reported recently (Levy *et al.,* 1996). The finding of only 5–10% of the total available CD4 positive T-cells (30–50%) in the PBMC cultures found to be replicating the large amounts of virus during the peak log and stationary phase cultures are intriguing and not readily explained. We are currently investigating both cellular and virus related mechanisms for this paradox.

As part of the quantitative studies performed in this kinetic study, infectious titers were determined during virus replication in both MDMs and PBMCs. Overall, HIV-1_{ADA}, whether derived from autologous MDMs or PBMCs, maintained tropism and replicated in both cell types. Notably, during viral replication in MDMs, peak infectious titers were found to occur at different times during the long log and stationary phases of viral replication. Infectivity for MDMs was observed first at Day 3 with maximal infectivity titers observed during late log phase (Day 14) of virus replication; whereas peak infectious titers on MDMs had fallen 1–2 logs (despite a similar average amount of virion-associated gp120 to be discussed later). In contrast, when HIV-1_{ADA} replicating in PBMCs was tested for infectivity during the same log and stationary phases in both PBMCs and MDMs, a lower infectivity titer was observed and ranged between 10^2 and 10^3 throughout the 14 days of sampling, with the peak infectivity (10^3) occurring at Day 6 for both MDMs and PBMCs.

To further characterize the qualitative and quantitative kinetics of viral replication, specifically infectivity and virus particle counts, comparisions of infectious titers and I/NI ratios during viral replication from primary human lymphoid cell types were made (Table 2). The I/NI ratio for HIV-$1_{ADA/MDM}$ replicating in MDMs increased during the transition from the log to the stationary phase, from a higher (i.e., 1:10^3 at Day 10) to a lower (1:10^7 at Day 20) value demonstrating a greater infectious fraction was produced earlier despite the fact that more virus was produced during the stationary, steady-state period. As much as a 4-log difference in the infectivity ratio was noted to occur in as little as a 3- to 4-day interval (i.e., Days 10–20, Table 2). In contrast, when the same inocula of HIV-$1_{ADA/MDM}$ were titered simultaneously back on the autologous donors PBMCs, a 2-log less I/NI ratio (1:10^5 at Day 10) was observed. This ratio increased later by greater than 1 log (1:10^4) on Day 17, and, however, returned to a new maximum value (1:10^8) on Day 24. These results demonstrate that during viral repliction from MDMs, a greater infectious fraction (100×) exhibited in HIV-$1_{ADA/MDM}$ during the log phase (Day 10) for MDMs than the same donor's PBMCs. Interestingly, virus obtained just 7 days later (i.e., stationary phase) exhibit a 10× decline in the I/NI ratio on MDMs, yet exhibited an increase in the infectious fraction (10×) for PBMCs. In comparision, the I/NI ratio for HIV-$1_{ADA/PBMC}$ replicating in PBMCs between 0 and 10 days demonstrated lower infectivity as exhibited by the lower and narrower I/NI ratio (1:10^5–10^6) or 1 infectious particle for every 1.0×10^5 to 1.0×10^6 particles. Similar kinetics and infectious ratios were also shown by other primary stocks made in the laboratory (unpublished data) and thus the present data may not merely be the results of a single experiment as performed in this study. The higher infectious fraction of HIV-$1_{ADA/MDM}$ replicating in MDMs *in vitro* would be consistent with the notion of virus initially selecting macrophage lineage cells located in strategic sites *in vivo* capable of producing more infectious cell-free virus for rapid dissemination. These findings may suggest one biological explanation for macrophage tropism of lentivirus and for previous reports demonstrating HIV-1 isolated from acute infections are generally monocytotropic (Schuitemake *et al.,* 1991; Roos *et al.,* 1992; Zhu *et al.,* 1993 and van't Wout *et al.,* 1994). In addition, some strains are believed to more efficiently infect and replicate in dendritic cells (Lanfhoff *et al.,* 1991; Cameron *et al.,* 1996) and Langerhans' cells derived from skin tissue (Soto-Ramirez *et al.,* 1996) than other cell types. Similar more quantitative autologous studies as described here will need to be done with these other cells types to confirm this hypothesis.

The extremely low infectious fraction when compared to the amount of virus produced daily on a per PBMC basis presented an interesting paradox. More specifically, during the initial infection of both primary cell types at a m.o.i. of 0.03, approximately 5.4×10^5 cells would have been infected. Given that the rate is only 5.3×10^3 particles per cell per day at peak production, only 2.9×10^9 virions total in the culture should have been produced in the first rounds of virus replication. At a I/NI ratio of 1:10^6–10^7, only 10^2–10^3 of these in total would be infectious. Thus, another 10^2 or 10^3 PBMCs would have been infected from the 2.9×10^9 cell-free particles. Experimentally, however, approximately up to 2.2×10^6 cells were assumed to be infected and lost by the end of the log/stationary phases (4 days total) (Fig. 2D). Thus the resulting productive infection of these additional 1.66×10^6 cells (the difference between 5.4×10^5 and 2.2×10^6) is up to 3 to 4 orders of magnitude greater than would have occurred by cell-free virus. Put in perspective, given the 2-day lag time and low cell-free infectivity, infection of these additional PBMCs by 2.9×10^9 virions would never have occurred. The apparent efficiency of HIV-$1_{ADA/MDM}$ infection in PBMCs, as characterized in this study by the short lag phase and rapid, high-titered log/stationary phase (a total of 6 to 10 days), despite the low I/NI ratio, strongly suggests that process(es) other than

cell-free transmission are operational (i.e., cell-to-cell transmission) (Gupta *et al.,* 1989). These findings would contribute to the neutralization resistance in PBMC, (Moore and Ho, 1995) and are consistent with two earlier reports demonstrating that cell-to-cell *in vitro* transmission was 10^2 to 10^3 times greater than cell-free virus *in vitro* using laboratory strains and human T-cell lines (Sato *et al.,* 1992; Dimitrov *et al.,* 1993). Further evidence for this increased efficiency was reported as measured by the accumulation of full length linear viral DNA which was found to occur within 15 min (Sato *et al.,* 1992). Likewise we have observed single infected H9 cells capable of fusing and infecting multiple neighboring cells within 30 min (data not shown). In contrast, the infection of MDMs appears to proceed by both cell-free and cell-to-cell spread. The former is evidenced by the lower I/NI ratio (higher infectious fraction $\sim$1:10^3) which permits the initially infected 4.5×10^5 cells (m.o.i. $\sim$0.03) to produce approximately 450,000 more infectious particles per day capable of infecting another 4.5×10^5 cells per day. The combination of a greater number of susceptible cells capable of producing a larger infectious fraction per replication cycle with a sustained capacity for viral replication allows for the infection to spread and be sustained by cell-free viruses *in vitro.* These results, if extrapolated *in vivo,* may suggest that early dissemination of HIV-1, if associated with cells of macrophage lineage, may occur as cell-free viruses after which the concomitant infection of PBMCs proceeds more efficient through cell-to-cell contact of the relatively more mobile lymphocyte pool (for a review see Pabst, 1988). If such a low infectivity is operational in CD4 lymphocytes *in vivo* (and it appears that it is; 10^5–10^7), the infection is unlikely to proceed via cell-free infectious viruses produced by $CD4^+$ lymphocytes. Thus, cell-to-cell transmission would generally be the case where the *in vivo* I/NI ratio was reported to range from 1:10^4 to 10^6 (Piatak *et al.,* 1993), but would not be operational in some cases where the ratio was 1:10^3 or higher (Piatak *et al.,* 1993; Perelson *et al.,* 1996).

Notably, the number of infectious particles produced by a single CD4 positive lymphocyte was less than 1 per cell per day (0.053 infectious units). This finding, when contrasted to the 10^2 infectious units per cell per 3 to 4 days reported previously (Dimitrov *et al.,* 1993) for the laboratory strain HIV-1_{NL4-3} replicating in the human T-cell line A3.01 (despite the production of similar numbers of virus particles), demonstrates the large differences which can exist *in vitro* depending on the virus and cell culture systems employed. This 10^2 to 10^3 log lower infectious fraction in PBMCs is closer to what may be observed *in vivo* where the I/NI ratio has been reported generally to range from 1:10^4 to 1:10^6 (Piatak *et al.,* 1993). Paradoxically, the general declining infectious fractions for HIV-$1_{ADA/MDM}$ and HIV-$1_{ADA/PBMC}$ (low I/NI ratios) occurred when the virus particle gp120 concentrations increased by 3 to 4 fold in the concentration of gp120 per virion over the preceding 3- to 4-day time intervals and suggests a rather rapid qualitative and quantitative change occurs in the virus population.

It is generally accepted that the viral surface molecule gp120 plays a role directly or indirectly in the initial stages of infection of host cells (Layne *et al.,* 1990; Wu *et al.,* 1995). Previous ultrastructural studies (for a review see Gelderblom, 1991) showed each virion with a full complement of gp120 on the surface possessed approximately 70 oligomers of 3 or 4 gp120 molecules for a total of 210 to 280 molecules per virus particle. In addition, this study and others, using laboratory strains, demonstrated that spontaneous shedding of the viral-associated gp120 occurs over time resulting in progressive loss of infectivity while at the same time increasing the susceptibility of virions to neutralization with antibody and/or sCD4 (Layne *et al.,* 1991, 1992). Typical acutely harvested stocks of HIV-1 laboratory strains generally were found to have 2–10 molecules of gp120 per virion (Layne *et al.,* 1992 and see Results). Interestingly, the averaged density of gp120 molecules per total virions for all viruses used in this study, regardless of the primary cells used to passage were about 10- to 25-fold higher than for the same laboratory strains HIV-1_{MN} grown in H9 cell line (see Results) and HIV-$1_{HXB3/H9}$ during a 48-hr period (Layne *et al.,* 1992). This finding suggests the concentrations of gp120 per virion for primary isolates and laboratory strains during a log/stationary phase growth cycle in primary cells result in increased amounts of gp120 in their envelopes. This study cannot, however, rule out that virus subpopulations in the HIV-1 stocks were not selected by the PBMCs which for whatever reason incorporated more gp120. In support of our primary findings and/or that more of the virion-associated gp120 is immunologically more accessible to our polyclonal antibody used in the ELISA, a similar result was reported for molecular clones of a laboratory strain and primary macrophage-tropic isolate. Using gp120/p24 ELISA data, the average density of gp120 spikes on a virion of the macrophage-tropic viruses was significantly greater ($\sim$4$\times$) than those of T-cell-line-adapted viruses (O'Brien *et al.,* 1994). These studies together provide direct evidence that primary isolates of HIV-1 derived from primary lymphoid cells appear to have greater amounts of gp120. This result could again contribute significantly to the general phenomena of neutralization resistance observed with primary isolates (Layne *et al.,* 1991, 1992; Stamatatos *et al.,* 1994; for a review see Moore and Ho, 1995).

As spontaneous shedding of the gp120 from HIV-1 has been well characterized, samples taken during the kinetic wash-out experiments allowed for a crude characterization of the spontaneous shedding rates of gp120 from HIV-1_{ADA} derived from MDMs and PBMCs. Interestingly, HIV-$1_{ADA/MDM}$ replicating in MDMs during the log phase exhibited no detectable shedding of the gp120 over two sampling intervals of 96 and 72 hr duration

between Days 10 and 17 of culture. This half-life appears to be prolonged when compared to the previous report in which a laboratory strain was found to shed the gp120 with a half-life of 30 hr (Layne *et al.,* 1991) and other preliminary data in the laboratory demonstrating that other primary isolates derived from PBMCs exhibited complete shedding of their envelopes between 10 to 72 hr (unpublished data). Thus it appears significant differences can exist for primary isolates depending on which primary cell types they are derived from. Reasons for these differences are unclear at this time; however, available evidence from the current study suggests that both the host cell and the virus may play a role in the shedding phenotype.

In conclusion, we have quantitatively and qualitatively characterized the initial lag, log-growth, and stationary phase kinetics of a dual-tropic primary HIV-1 in both MDMs and PBMCs in terms of various biological and bio-physicochemical parameters. By the use of an autologous donor cell assay, a kinetic wash-out experimental format and particle separation techniques, the parameters that have been determined include viral replication kinetics, viral production rate per cell, soluble and virion-associated virus-encoded gene products (such as gp120 concentrations, RT, and p24), the numbers and stability of gp120 molecules associated with a virion, the ratios of infectious to noninfectious virus particles and reciprocal infectivity/tropism. These new insights should be useful for future studies aimed at characterization of primary clinical isolates of HIV-1 and further our understanding of how they may impact on the disease process itself.

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REFERENCES

Arthur, L. O., Bess, J. W., Sowder, R. C., II, Benveniste, R. E., Mann, D. L., Chermann, J.-C., and Henderson, L. E. (1992). Cellular protein bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* 258, 1935–1938.

Cameron, P. C., Lowe, M. G., Sotzik, F., Coughlan, A. F., Crowe, S. M., and Shortman, K. (1996). The interaction of macrophage and non-macrophage tropic isolates of HIV-1 with thymic and tonsillar dendritic cells in vitro. *J. Exp. Med.* 183, 1851–1856.

Cantin, R., Fortin, J.-F., and Tremblay, M. (1996). The amount of host HLA-DR proteins aquired by HIV-1 is virus strain and cell type-specific. *Virology* 218, 372–381.

Chun, T. W., Finzi, D., Margolick, J., Chadwick, K., Schwartz, D., and Siliciano, R. F. (1995). *In vitro* fate of HIV-1-infected T cells: Quantitative analysis of the transition to stable latency. *Nature Med.* 1, 1284–1290.

Dimitrov, D. S., Willey, R. L., Sato, H., Chang, L.-J., Blumenthal, R., and Martin, M. A. (1993). Quantitation of human immunodeficiency virus type 1 infection kinetics. *J. Virol.* 67, 2182–2190.

Gelderblom, H. R. (1991). Assembly and morphology of HIV-1: Potential effect of structure on viral function. *AIDS* 5, 617–638.

Gendelman, H. E., Orentein, J. M., Martin, M. A., Ferruca, C., Mitra, R., Phipps, T., Wahl, L. A., Lane, H. C., Fauci, A. S., and Burke, D. S. (1988). Efficient isolation and propagation of human immunodeficiency virus on recombinant colony stimulating factor 1-treated monocytes. *J. Exp. Med.* 167, 1428–41.

Gupta, P., Balachandran, R., Ho, M., Encrio, A., and Rinaldo, C. R. (1989). Cell to cell transmission of human immunodeficiency virus Type I in the presence of azidothymidine and neutralizing antibody. *J. Virol.* 63, 2361–2365.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 373, 123–126.

Langhoff, E., Terwilliger, E. F., Bos, H. J., Kalland, K. H., Poznansky, M. C., Bacon, O. M. L., and Haseltine, W. A. (1991). Replication of human immunodeficiency virus type 1 in primary dendritic cultures. *Proc. Natl. Acad. Sci. USA* 88, 7998–8002.

Layne, S. P., Merges, M. J., Dembo, M., Spouge, J. L., and Nara, P. L. (1990). HIV requires multiple gp120 molecules for CD4-mediated infection. *Nature* 346, 277–279.

Layne, S. P., Merges, M. J., Spouge, J. L., Dembo, M., and Nara, P. L. (1991). Blocking of human immunodeficiency virus infection depends on cell density and viral stock age. *J. Virol.* 65, 3293–3300.

Layne, S. P., Merges, M. J., Dembo, M., Spouge, J. L., Conley, S. R., Moore, J. P., Raina, J. L., Renz, H., Gelderblom, H. R., and Nara, P. L. (1992). Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. *Virol.* 189, 695–714.

Levy, J. (1993). Pathogenesis of human immunodeficiency virus infection. *Microbiol. Rev.* 57, 183–289.

Levy, J. A., Ramachandran, B., and Barker, E. (1996). Plasma viral load, $CD4^+$ cell counts, and HIV-1 production by cells. *Science* 271, 670–671.

Lin, H. J., Myers, L. E., Yen-Lieberman, B., Hollinger, F. B., Hemard, D., Hooper, C. J., Kokka, R., Kwok, S., Rasheed, S., Vahey, M., Winters, M. A., McQuay, L. J., Nara, P., Reichelderfer, P., Cooms, R. W., and Jackson, J. B. (1994). Multicenter evaluation of quantification methods for plasma human immunodeficiency virus tyupe 1 RNA. *J. Infect. Dis.* 170, 553–562.

McDougal, J. S., Cort, S. P., Kennedy, M. S., Cabridilla, C. D., Feorino, P. M., Frances, D. P., Hicks, D., Kalyanaraman, V. S., and Martin, L. S. (1985). Immunoassay for the detection and quantitation of infectious human retrovirus, lymphoadenopathy-associated virus (LAV). *J. Immunol. Methods* 76, 171–183.

Montefiori, D. C., Cornell, R. J., Zhou, J. Y., Zhou, J. T., Hirsch, V. M., and Johnson, P. R. (1994). Complement control proteins, CD46, CD55, and CD59, as common surface constituents of human and simian immunodeficiency viruses and possible targets for vaccine protection. *Virology* 205, 82–92.

Moore, J. P., and Jarrett, R. F. (1988). Sensitive ELISA for the gp120 and gp 160 surface glycoproteins of HIV-1. *AIDS Res. Hum. Retroviruses* 4, 369–379.

Moore, J. P., Wallace, L. A., Follett, E. A. C., and McKeating, J. A. (1989). An enzyme-linked immunosorbent assay for antibodies to the envelope glycoproteins of divergent strains of HIV-1. *AIDS* 3, 155–163.

Moore, J. P., and Ho, D. D. (1995). HIV-1 neutralization: The consequences of viral adaptation to growth in transformed T-cells. *AIDS* 9, S117–S136.

Nara, P. L., Hatch, W. C., Dunlop, N. M., Robey, W. G., Arthur, L. O., Gonda, M. A., and Fischinger, P. J. (1987). Simple, rapid, quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. *AIDS Res. Hum. Retroviruses* 3, 283–302.

Nara, P. L., Layne, S. P., Garrity, R. R., and Goudsmit, J. (1991). Neutralization of HIV-1: A paradox of humoral proportions. *FASEB J.* 5, 2437–2455.

Nara, P., Tsai, W.-P., Kung, H. F., Minassian, A., Garrity, R., Goudsmit, J., and Rimmelzwaan, G. (1992). Universal cellular tropism? *Nature* 360, 215–216.

O'Brien, W. A., Mao, S.-H., Cao, Y., and Moore, J. P. (1994). Macrophage-tropic and T-cell line-adapted chimeric strains of human immunodeficiency virus type 1 differ in their susceptibilities to neutralization by soluble CD4 at different temperatures. *J. Virol.* 68, 5264–5269.

Orentas, R. J., and Hildreth, J. E. K. (1993). Association of host cell surface adhesion receptors and other membrane proteins with HIV and SIV. *AIDS Res. Hum. Retrovirus* 11, 1157–1165.

Pabst, R. (1988). The spleen in lymphocyte migtation. *Immunol. Today* 9, 43–45.

Perelson, A. S., Neumann, A. V., Markovitz, M., Leonard, J. M., and Ho, D. D. (1996). HIV-1 dynamics *in vivo:* virion clearance rate, infected cell life-span, and viral generation time. *Science* 271, 1582–1586.

Piatak, M., Jr., Saag, M. S., Yang, L. C., Stark, S. J., Kappes, J. C., Luk, K. C., Hahn, B. H., Shaw, G. M., and Lifson, J. D. (1993). High levels of HIV-1 in plasma during all stages of infection determine by competitive PCR. *Science* 259, 1749–1754.

Roos, M. The. L., Lange, J. M. A., DeGoede, R. E. Y., Coutinho, R. A., Schellkens, P. The. A., Miedema, F., and Tersmette, M. (1992). Virus phenotype and immune response in primary human immunodeficiency virus type 1 (HIV-1) infection. *J. Infect. Dis.* 165, 427–432.

Saifuddin, M., Parker, C., Peeples, M. E., Gorny, M. K., Zolla-Pazner, S., Ghassemi, M., Rooney, I. A., Atkinson, J. P., and Spear, G. T. (1995). Role of virion associated glycosylphosphatidylinositol-linked CD55 and CD59 in complement resistance of cell line-derived and primary isolates of HIV-1. *J. Exp. Med.* 182, 501–509.

Sato, H., Orenstein, J. M., Dimitrov, D., and Martin, M. (1992). Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. *Virology* 186, 712–724.

Schuitemake, H., Kootstra, N. A., DeGuede, R. E., DeWolf, F., Miedema, F., and Tersmette, M. (1991). Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture. *J. Virol.* 65, 356–363.

Soto-Ramirez, L. E., Renjifo, B., McLane, M. F., Marlink, R., O'Hara, C., Sutthent, R., Wasi, C., Vithayasai, P., Vithayasai, V., Apichartpiyakul, C., Auewarakul, P., Pena Cruz, V., Chui, D.-S., Osathanondh, R., Mayer, K., Lee, T.-H., and Essex, M. (1996). HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV-1. *Science* 271, 1291–1293.

Stamatatos, L., Werner, A., and Cheng-Mayer, C. (1994). Differential regulation of cellular tropism and sensitivity to soluble CD4 neutralization by the envelope gp120 of human immunodeficiency virus type 1. *J. Virol.* 68, 4973–4979.

Tsai, W.-P., Hirose, K., Nara, P. L., Kuang, Y.-d., Conley, S., Li, B.-Q., Kung, H.-F., and Matsushima, K. (1991). Decrease in cytokine production by HIV-infected macrophages in response to LPS-mediated activation. *Lymphokine Cytokine Res.* 10, 421–429.

Tsai, W.-P., Kung, H. F., Goudsmit, J., Rimmelzwaan, G., Minassian, A., Garrity, R., and Nara, P. (1992). HIV-1 tropism: Truth or consequences? *AIDS Res. Hum. Retroviruses.* 8, 1749–1750. [Letter to the Editor]

Volskey, D. J., Pellegrino, M. G., Li, G., Logan, K. A., Aswell, J. E., Lawrence, N. P., and Deker, S. R. (1990). Titration of human immunodeficiency virus type 1 (HIV-1) and quantitative analysis of virus expression *in vitro* using RNA-RNA hybridization. *J. Virol. Methods* 28, 257–272.

van't Wout, A. B., Kootstra, N. A., Mulder-Kampinga, G. A., Albrecht-van Lent, N., Scherpbier, H. J., Veenstra, J., Boel, K., Coutinho, R. A., Miedema, F., and Schuitemaker, H. (1994). Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J. Clin. Invest.* 94, 2060–2067.

Wei, X., Ghosh, S. K., Taylor, M. J., Johnson, V. A., Emini, E., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Saag, M. S., and Shaw, G. M. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373, 117–122.

Weiss, R. A. (1993). How does HIV cause AIDS? *Science* 28, 1273–1278.

Wong-Staal, F., Shaw, G. M., Hahn, B. H., Salahuddin, S. Z., Popovic, M., Markham, P., Redfield, R., and Gallo, R. C. (1985). Genomic diversity of human T-lymphotropic virus type III (HTLV-III). *Science* 229, 759–762.

Wu, S.-C., Spouge, J. L., Conley, S. R., Tsai, W.-P., Merges, M. J., and Nara, P. L. (1995). Human plasma enhances the infectivity of primary human immunodeficiency virus type 1 isolates in peripheral blood mononuclear cells and monocyte-derived macrophages. *J. Virol.* 69, 6054–6062.

Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A., and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. *Science* 261, 1179–1181.